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IN RE APPLICATION OF	: Frank Austrup and Michael Giesing
SERIAL NO.	: 09/744,866
FILED	: April 2, 2001
FOR	: Cancer cells from body fluids containing cells, isolation thereof and agents containing same

DECLARATION UNDER 37 C.F.R. §1.132

COMMISSIONER OF PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450

SIR:

Now comes Prof. Dr. med. Michael Giesing who deposes and states:

1. I am a graduate of University of Bonn, Germany, and received my doctorate degree in the year 1971.

2. I have been working for 14 years as a laboratory physician in the field of molecular oncology.

3. I have read and fully understood U.S. application, Ser. No. 09/744,866.

4. I have read and fully understood the Office Action of July 23, 2004 and the prior art cited therein.

5. The following experiments and investigations were carried out by me or under my direct supervision.

For more than a decade I have been using the molecular characterization of disseminated cancer cells as a diagnostic tool that allows an "online approach" to cancer management. There is now overwhelming evidence that this approach provides more information about metastatic potential, drug sensitivity and the development of therapy resistance than the analysis of the primary tumor and helps identifying subgroups of patients who might receive benefit from a particular therapy. Additionally, after removal of the primary tumor, the disseminated cancer cells and metastases derived therefrom represent the therapeutic target. The emergence of new drugs directed against specific cancer-related molecules makes it mandatory to stratify treatment accordingly in order to properly identify molecular targets, thus opening further applications for the characterization of disseminated cancer cells.

One avenue to detect and/or characterize disseminated cancer cells is based on immunological techniques, especially the use of paramagnetic beads conjugated to monoclonal antibodies to capture disseminated cells by virtue of antigens expressed on disseminated cancer cell surface. Disadvantageously, in these methods, crosslinking of the surface antigens can cause unpredictable effects such as apoptosis, anergy, activation and other changes in the state of the cells. Such effects can drastically change what is determined on subsequent characterization of isolated disseminated cancer cells. For example, the expression profile of a cell may be affected within a few minutes. It is impossible in such cases to rule out that the data obtained in this way reflect apparent properties which the disseminated cancer cells in the body fluid did not have prior to their isolation. As a further disadvantage, the adhering antibodies can be removed only with

unfavorable consequences for the cell or not at all. If the antibodies are directed against intracellular commponents, fixation and perforation of the cell are necessary resulting in cell death. In these circumstances, bioassays involving living and proliferative cells, are very difficult or even impossible. A further disadvantage of purification via antibodies is cross-reactivity of epitopes, so that normal cells may also be isolated.

The prior art method relying on use of antibody-conjugated magnetic beads prior to a filtration step that retains cells attached to immunobeads on a filter, would have the same deficiencies as in the case where the filtering step would be omitted because it is the use of antigen-specific immunoadsorption that affects the properties of the cancer cells thus isolated.

The method disclosed in U.S. application, Ser. No. 09/744,866 circumvents these disadvantages and, moreover, has further advantages which will be apparent from the following experiments and investigations.

<u>5.1 Using screens of different pore width</u>

Mononuclear cells were obtained from blood samples of a number of cancer patients by density gradient centrifugation and then subjected to the screening process as described in example 1 of U.S. application, Ser. No. 09/744,866. Screens with a pore width of 200 μm, 115 μm, 74 μm, 51 μm, 38 μm, 27 μm and 20 μm, were used. The filtrate which passed the screen with a pore width of 20 μm and all retentates obtained were assessed for various RNAs and DNAs, for instance, cytokeratin 20 (CK20)-mRNA expression and gastric

inhibitory peptide (GIP)-mRNA expression, both widely accepted markers for blood borne cancer cells. To this end, RNA was extracted from the filtrate or retentates using commercially available RNA purification kits according to the manufacture's instructions. The experimental protocol for measuring CK20-mRNA expression is described in Exhibit A attached hereto and that for measuring GIP-mRNA is described in Exhibit B attached hereto.

The CK20-mRNA expression as found is shown in the following table I (the lower the value, the higher the content of CK20-mRNA positive cells; "-" means no significant CK20-mRNA expression):

Table I:

Pore size (µm)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
200	-	-	36.790	-	-	-	42.526
115	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-
51	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-
27	-	-	48.214	36.905	-	41.600	-
20	44.103	18.588	31.476	35.982	-	-	-
Filtrate	39.020	-	38.831	38.270	36.083	-	-

Further, the GIP-mRNA expression as found is shown in the following table II ("neg" means no significant GIP-mRNA expression; "pos" means significant GIP-mRNA expression; "++" or "+++" means strong or very strong GIP-mRNA expression; "nd" means not determined):

Table II:

Pore size (µm)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
200	neg	neg	neg	neg	neg	pos	neg
115	neg	neg	pos	neg	neg	neg	neg
74	neg	+++	neg	neg	neg	neg	neg
51	neg	neg	neg	neg	neg	neg	neg
38	neg	neg	neg	neg	neg	neg	neg
27	neg	++	neg	neg	neg	neg	neg
20	neg	++	neg	neg	neg	pos	pos
Filtrate	neg	neg	neg	neg	neg	neg	neg

The method used for isolating disseminated cancer cells is based on the finding that disseminated cnacer cells in blood or bone marrow have a larger size than non-cancer cells in blood or bone marrow. Thus, two opposite effects have to be considered. On the one hand, the larger the diameter of the pores, the less disseminated cancer cells will be retained on the screen. On the other hand, the smaller the diameter of the pores, the more non-cancer cells will be retained on the screen and thus reduce the cancer cell proportion in the isolated cancer cell fraction.

As can be seen from the above tables I and II, both CK20-mRNA positive and GIP-mRNA positive cells were retained on screens having relatively small pores of 27 µm and lower. Moreover, within this lower range a further distinction can be made between those cells which were retained on a screen having pores of 27 µm, those which were retained on a screen having pores of 20 µm (but pass the screen having pores of 27 µm) and/or those which may be retained on a screen having pores of less than 20 µm (but pass the screen having pores of 20 m). This means that using a screen having pores of 27 µm allowed isolation of CK20-mRNA positive or GIP-mRNA positive cells in certain cases (here patients 3, 4 and 6 for CK20 or patient 2 for GIP, respectively) but failed in other cases

(here patients 1, 2 and 5 for CK20 or patients 6 and 7 for GIP, respectively). On the other hand, using a screen of 20 µm was more effective in yielding CK20-mRNA positive cells (here in addition to patients 3, 4, 6 also in patients 1 and 2) and also in yielding GIP-mRNA positive cells (here in addition to patient 2 also in patients 6 and 7).

Merely the CK20-positive cancer cells of patient 5 passed the screen of 20 µm. These CK20-positive cancer cells may possibly be retained on a screen having pores in the range of 15 µm (the lower limit of the claimed range) to 20 µm. However, this has not been tested. Since the degree of cancer cell enrichment decreases with decreasing pore diameter, with screens having pores of less than 15 µm the problem arises that a relatively high proportion of non-cancer cells will be retained on the screen.

In light of these experiments I reasonably expect screens having a pore width below or above 20 µm (and within the range of 15 µm to 30 µm) to work, although less effectively than a screen having a pore width of about 20 µm .

5.2 Prognostic meaning of different types of blood borne cancer cells

Blood samples from several breast cancer patients were investigated.

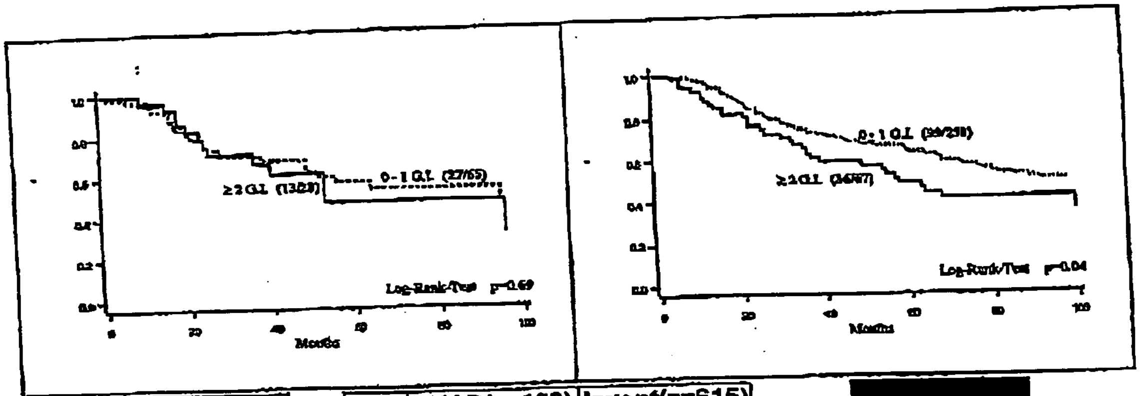
From each sample, a cell suspension containing mononuclear cells (MNC suspension) was prepared as described in example 1 of U.S. application, Ser. No. 09/744,866 (these cells serve as control); disseminated cancer cells from were isolated as described in example 1 of U.S. application, Ser. No. 09/744,866 (these cells are hereinafter

referred to as M1 cells); and disseminated cancer cells from blood samples from breast cancer patients were isolated by conventional immunomagnetic separation as described in Exhibit C attached hereto (these cells are hereinafter referred to as M0 cells).

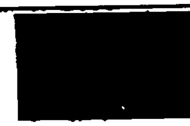
Genomic DNA was extracted from the isolated cells using commercially available DNA purification kits (Qiagen, Germany).

Then, the DNA extracted from the M0 cells (for each of a total of 169 breast cancer patients) and the DNA extracted from the M1 cells (for each of a total of 615 breast cancer patients) were assessed for 8 different DNAs, as described in Exhibit D attached hereto. The percentages of patients with M0 cells having 0, 1, 2, or at least 3 genomic imbalances were determined, and the corresponding percentages were also determined for the M1 cells. The results are displayed in the following figure 1, wherein the table states said percentages for M0 cells (see the left-hand column titled "bead") and for M1 cells (see the right-hand column titled "invent").

Figure 1:



G.I.	BEAD(n=169)	Invent(n=615)
0	42%	52%
1	43%	31%
2	17%	11%
≥3	8%	7%



As can be seen, in both cases the cells could be identified as cancer cells due to the presence of genomic imbalances (DNA amplifications and LOHs).

Then, the prognostic meaning of the M1 cells and M0 cells was compared. To this end, the patients' follow-up was done by specialized oncologists and detailed patients' data (diagnosis, histology, treatment regimens, clinical follow-up data) were provided by means of a questionnaire. Relapse-free survival interval was defined as the period between date of first diagnosis of breast cancer and documented relapse. Survival curves were drawn with the use of the Kaplan-Meyer product limit method. The log-rank test was used to compare two or more survival distributions. All computations were performed using the SAS software package, release 6.12.

Figure I shows actual relapse-free survival curves for said breast cancer patients stratified into 2 groups, i. e. those with 0 or 1 genomic imbalance and those with at least two genomic imbalances in M0 cells (left-hand Kaplan-Meyer diagram) and in M1 cells (right-hand Kaplan-Meyer diagram).

As shown in Figure 1, in the M0 cells an increased number of genomic imbalances did not correlate with a higher probability of disease recurrence whereas in M1 cells an increased number of genomic imbalances did in fact correlate with a higher probability of disease recurrence. Thus, M0 cells do not have the prognostic value of the M1 cells.

Based on the results obtained, I can hypothesize that at least two types of disseminated cancer cells occur in blood and bone marrow:

- Epithelial, small cancer cells (mean diameter below 20 μm)
- Mesenchymal, large cancer cells (mean diameter above 20 μm) which are also referred to as micrometastases.

In both types of cancer cells similar oncogenic DNA aberrations can be found which are not present in corresponding non-cancer cells. However, as far as the clinical outcome (in particular the occurrence of metastases) is concerned, in most if not all tumors only the large cancer cells are premetastatic and therefore correlate with the clinical outcome of the cancer disease.

Actually, the risk of developing metastases seems to depend from the balance

between said small cancer cells and said large cancer cells. I have found, for instance, that successful chemotherapies can involve an epithelial-mesenchymal reversion, i. e., said balance is shifted towards the small cancer cells. In contrast thereto, I know of many cases in which chemotherapies even worsened the situation by shifting said balance towards the large cancer cells. This corresponds to a progression of the disease. Additionally, chemotherapy resistance genes detected in said large cancer cells worsened the probability for clinical tumor recurrence. Antihormonal therapies, however, e.g. in breast cancer patients, improved relapse free survival through the induction of the progesterone receptor in small cancer cells. In summary, drugs with different mechanisms of action target preferentially either said larger cancer cells or said epithelial, smaller cancer cells.

5.3 Prognostic meaning of cancer cells from ascitic fluid

A female patient (53 years) with a diagnosis of ovarian carcinoma and metastatic spread into the peritoneal cavity was examined. An ovarian tumor tissue sample, an ascitic fluid sample and a blood sample from said patient was provided. DNA and RNA was extracted from the tissue sample as described in Exhibit E attached hereto and from the ascitic fluid sample as described in Exhibit F attached hereto. MNCs and disseminated cancer cells were isolated from the blood sample, as described in example 1 of U.S. application, Ser. No. 09/744,866. The resulting preparations were then assessed for various DNAs and RNAs, as described in Exhibit G attached hereto. The results obtained are shown in the following table III: ("-" means a negative result; "+/-" means a slightly positive result; "+" means a positive result; "++" means a strongly positive result; "+++" means a very strongly positive result)

Table III:

Gene Target	Ovarian Tissue	Ascitic Fluid	Disseminated Cancer cells
SOD	-	-	+++
TXNRD1			++
GPX1			+++
MDR1 ampl.	-	-	+
MDR1	-	-	+
MRP	-	-	-
Bcl-2	-	-	-
ß-Tub mt	-	-	+
Topo II	+/-	+/-	+
TS	-	-	+
GST	-	-	-
GCS	-	-	+
DCK			+
ER	+	+	+
PR	-	-	+
EGF-R	+	+	-
RFT	+	+	-
Erb B2 wt	+++	+++	-
Erb B2 splice	+++	++	-

Based on the above results, the geno- and phenotyping of ovarian tumor tissue, tumor cells from ascitic fluid, and disseminated cancer cells isolated from blood using the method described in U.S. application, Ser. No. 09/744,866, clearly revealed that tumor cells from ovarian tumor tissue and from ascitic fluid showed a similar geno- and phenotype which, in turn, is markedly different from the geno- and phenotype of the disseminated cancer cells.

Since the geno- and phenotype of said cells from ascitic fluid is still similar to the geno- and phenotype of the ovarian tumor tissue I would consider the tumor cells from ascitic fluid to belong to the class of M0 cells rather than to the class of M1 cells. It is against this background that I expect the cells from ascitic fluid for the patient examined

above not to have the prognostic meaning the disseminated cancer cells isolated from blood have.

7. The undersigned petitioner declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

8. Further deponent saith not.

Lienen, Germany, ... 14th 2005 ....

Attachments:

Exhibits A, B, C, D, E, F, G

Exhibit A

Detection of CK20-mRNA

The amounts of expressed cytokeratine 20 (CK20)-mRNA were determined by quantitative RT-PCR. The PCR format was based on the 5'-exonuclease assay known per se (TaqMan®) and was suitable for use on the TaqMan® 7700 sequence detector from Applied Biosystems (ABI).

More specifically, a RT-mix containing first strand buffer, dithiothreitol, RNA guard, random hexamers, dNTPs and reverse transcriptase was prepared. Then, the sample RNA was denatured and immediately cooled on ice, mixed free of air bubbles with the RT-mix, incubated to allow reverse transcription, incubated at higher temperature to stop reverse transcription, immediately cooled on ice and the resulting cDNA either subjected directly to PCR or frozen at –20 °C.

A PCR reaction mixture containing TaqMan® buffer, $MgCl_2$, dNTPs, CK20-specific sense primer, CK20-specific antisense primer, CK20-specific TaqMan® probe and heatstable polymerase was prepared. To this PCR reaction mixture was added the cDNA obtained by reverse transcription and the resulting mixture was subjected to PCR carried out in the ABI 7700 sequence detector (TaqMan®).

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to CK20-mRNA was present in the sample.

Exhibit B

Detection of GIP-mRNA

The amounts of expressed gastric inhibitory polypeptide (GIP)-mRNA were determined by quantitative RT-PCR. The PCR format was based on the 5'-exonuclease assay known per se (e.g. TaqMan®) and was suitable for use on the TaqMan® 7700 sequence detector from Applied Biosystems (ABI).

More specifically, a RT-mix containing first strand buffer, dithiothreitol, RNA guard, random hexamers, dNTPs and reverse transcriptase was prepared. Then, the sample RNA was denatured and immediately cooled on ice, mixed free of air bubbles with the RT-mix, incubated to allow reverse transcription, incubated at higher temperature to stop reverse transcription, immediately cooled on ice and the resulting cDNA either subjected directly to PCR or frozen at −20 °C.

A PCR reaction mixture containing TaqMan® buffer, $MgCl_2$, dNTPs, GIP-specific sense primer, GIP-specific antisense primer, GIP-specific TaqMan® probe and heatstable polymerase was prepared. To this PCR reaction mixture was added cDNA obtained by reverse transcription and the resulting mixture was subjected to PCR carried out in the ABI 7700 sequence detector (TaqMan®).

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to GIP-mRNA was present in the sample.

Exhibit C

Isolation of epithelial cancer cells from blood samples

A cell suspension containing mononuclear cells (MNC suspension) from blood samples was prepared as described in example 1 of U.S. application, Ser. No. 09/744,866. Anti-epithelial beads (40 µl/0.5 ml; anti-EGP beads, Dynal) were washed twice with 800 µl of PBS containing 0.2% BSA and 1 mM EDTA in a Dynal MPC-M magnetic strip (MPC for short). 40 µl of washed beads per 0.5 ml of MNC suspension were placed in an Eppendorf tube, the tube was rotated in a rotor at 4°C for 25 min and then placed in a tube stand, and the suspension from the tube lead was put into the tube.

The tube was placed in the MPC for 1 min and the cell suspension was discarded. Then, the magnetic strip was removed (or the tube was taken out) and addition of 800 µl of PBS containing 0.2% BSA and 1 mM EDTA was followed by cautious resuspension. These sequence of steps was repeated 6 times, finally resuspending in PBS/1 mM EDTA (without BSA).

The tube was again placed in the MPC for 1 min, and the supernatant was completely removed. The resulting beads with adherent cells were taken as cancer cell fraction (M0 cells).

For RNA/DNA isolation, the beads with adherent cells were resuspended in 200 µl of Trizol® (GIBCO) and stored at –80°C.

Exhibit D

Genomic imbalances include the loss of chromosome sections (which can be assessed by the loss of heterocygosity (LOH)) or gene amplifications.

The LOH-analyses of p53, Rb, DCC and/or APC alleles were carried out as described in Example 3 of U.S. application, Ser. No. 09/744,866, with the exception that, instead of a CD45 positive cell fraction (Example 2 of U.S. application, Ser. No. 09/744,866), a MNC fraction obtained as described in Example 1 of U.S. application, Ser. No. 09/744,866 was used as control. In an analogous manner, LOH-analyses of the microsatellite markers D11S528, D17S695 and D18S849 were carried out.

The amplification analyses of erb-B2 and c-myc were carried out as described in Example 4 of U.S. application, Ser. No. 09/744,866, with the exception that, instead of CD45 positive cell fraction (Example 2 of U.S. application, Ser. No. 09/744,866), a MNC fraction obtained as described in Example 1 of U.S. application, Ser. No. 09/744,866 was used as control.

Exhibit E

Isolation of DNA and RNA from ovarian tumor tissue sample

A piece of approximately 30 – 100 mg ovarian tumor tissue was placed in a tissue douncer and 1 ml Trizol® (GIBCO) was added. Then the tissue was minced with a glas pestle. The resulting solution was placed in an Eppendorf tube and stored at –80°C until RNA or DNA was isolated as usual.

The same proceudre was followed for isolating DNA and RNA form a normal ovarian tissue sample (which served as control).

Exhibit F

Isolation of DNA and RNA from ascitic fluid

Ascitic fluid was centrifuged at 400g at room temperature for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml PBS containing 0.2% BSA and 1 mM EDTA. 10 % FC blocking agent (huIgG 1 mg/ml) was added to the ascitic cell supension.

Anti-CD45 beads (anti-CD45 beads, Dynal) were washed twice with 800 µl of PBS containing 0.2% BSA and 1 mM EDTA in a Dynal MPC-M magnetic strip (MPC for short). 50 – 100 µl of washed beads per 1.0 ml ascitic cell suspension were placed in an Eppendorf tube, the tube was rotated in a rotor at 4°C for 20 min and then placed in a tube stand, and the suspension from the tube lead was put into the tube.

The tube was placed in the MPC for 1 min. The supernatant was placed in another Eppendorf tube and PBS containing 0.2% BSA and 1 mM EDTA was added until a total volume of 10 ml. This cell suspension was used for isolating cancer cells as described below.

The magnetic strip was removed (or the tube was taken out) and addition of 800 µl of PBS containing 0.2% BSA and 1 mM EDTA was followed by cautious resuspension. The last sequence of steps was repeated 3 times, finally resuspending the beads with adherent in PBS/1 mM EDTA (without BSA).

The tube was again placed in the MPC for 1 min, and the supernatant was completely removed. The resulting beads with adherent cells were used as control. For RNA/DNA isolation, the cells were added to 0.4 µl Trizol® (GIBCO).

The cell suspension (which contained the cancer cells) obtained above was subjected to the filtration procedure as described on Example 1 of U.S. application, Ser. No. 09/744,866 and the resulting Trizol® solution (containing the cells retained on the screen) was subjected to DNA/RNA isolation, as required.

Exhibit G

1. Detection of SOD-mRNA; TXNRD1-mRNA; GPX1-mRNA

The amounts of expressed manganese superoxide dismutase (SOD)-, thioredoxin reductase 1 (TXNRD1)- or glutathione peroxidase 1 (GPX1)-mRNA were determined by quantitative RT-PCR. The PCR format was based on the 5'-exonuclease assay known per se (TaqMan®) and was suitable for use on the TaqMan® 7700 sequence detector from Applied Biosystems (ABI).

More specifically, a RT-mix containing first strand buffer, dithiothreitol, RNA guard, random hexamers, dNTPs and reverse transcriptase was prepared. Then, the sample RNA was denatured and immediately cooled on ice, mixed free of air bubbles with the RT-mix, incubated to allow reverse transcription, incubated at higher temperature to stop reverse transcription, immediately cooled on ice and the resulting cDNA either subjected directly to PCR or frozen at –20 °C.

A PCR reaction mixture containing TaqMan® buffer, $MgCl_2$, dNTPs, specific sense primer, specific antisense primer, specific TaqMan® probe and heatstable polymerase was prepared. To this PCR reaction mixture was added the cDNA obtained by reverse transcription and the resulting mixture was subjected to PCR carried out in the ABI 7700 sequence detector (TaqMan®). A corresponding PCR was carried out using GAPDH-specific sense primer, GAPDH-specific antisense primer and GAPDH-specific TaqMan® probe

The following SOD-specific primers and probes were used (MNSOD, SOD2; accession No.: M36693):

sense: 5'-GTCACCGAGGAGAAGTACCAGG -3'

antisense: 5'-GGGCTGAGGTTTGTCCAGAA-3'

probe: 5'-CGTTGGCCAAGGGAGATGTTACAGCCC-3'

Size of the PCR product: 131 bp.

The following TXNRD1-specific primers and probes were used (TXNRD1; accession No.: X91247 cDNA):

sense: 5'-GGAGGGCAGACTTCAAAAGCTAC-3'

antisense: 5'-ACAAAGTCCAGGACCATCACCT-3'

probe; 5'-TTGGGCTGCCTCCTTAGCAGCTGCCA-3'

Size of the PCR product: 158 bp.

The following GPX1-specific primers and probes were used (GPX1; accession No.: M21304):

sense: 5'-CTCGGCTTCCCGTGCAA-3'

antisense: 5'-TGAAGTTGGGCTCGAACCC-3'

probe: 5'-AGTTTGGGCATCAGGAGAACGCCAAGAA-3'

Size of the PCR product: 109 bp.

The following GAPDH-specific primers and probes were used (GAPDH; accession No. X01677):

sense: 5'-TGCTGATGCCCCCATGTTC-3'

antisense: 5'-GGCAGTGATGGCATGGACTG-3'

probe: 5'-TCAAGATCATCAGCAATGCCTCCTGCA-3'

Size of the PCR product: 174 bp.

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to the relevant mRNA was present in the sample.

The progress of the PCR was measured on line. The curve recorded for the progress of the reaction served as a basis for determining the amount of cDNA amplified. The earlier the reaction changes to the exponential phase (threshold value CT), the more cDNA corresponding to the relevant mRNA was present in the sample.

For the evaluation, the ratio of cell equivalents of the relevant mRNA to cell equivalents of GAPDH-mRNA was found for the cancer cell fraction and for the control cell fraction, and the ratio of the resulting quotients was found in turn. A ratio (cancer cells/control cells) significantly higher than 1 was considered as a positive result representing an overexpression of the relevant mRNA in the cancer cell fraction.

2. Detection of MDR1-, MRP-, Bcl-2-, Topo II-, TS-, GST-, GCS-, DCK-, ER-, PR-, EGF-R-, RFT-, Erb B2 (wildtype) and Erb B2 (splice)-mRNA

The amounts of expressed MDR1-, MRP-, Bcl-2-, Topo II-, TS-, GST-, GCS-, DCK-, ER-, PR-, EGF-R-, RFT-, Erb B2 (wildtype) and Erb B2 (splice)-mRNA were determined by quantitative RT-PCR as described above for SOD-, TXNRD1- or GPX1-mRNA.

For the evaluation, the ratio of cell equivalents of the relevant mRNA to cell equivalents of GAPDH-mRNA was found for the cancer cell fraction and for the control cell fraction, and the ratio of the resulting quotients was found in turn. For MDR1-, MRP-, Bcl-2-, TS-, GST-, GCS-, DCK-, ER-, PR-, EGF-R-, RFT-, Erb B2 (wildtype) and Erb B2 (splice)-mRNA a ratio (cancer cells/control cells) significantly higher than 1 was considered as a positive result representing an overexpression of the relevant mRNA in the cancer cell fraction. For Topo II-mRNA, a ratio (cancer cells/control cells) significantly lower than 1 was considered as a positive result representing an underexpression of the relevant mRNA in the cancer cell fraction.

3. *Detection of mdr1 ampification*

Whether or not there was an amplification of the mdr1 gene in the cancer cell fraction was determined by PCR-based coamplification of the mdr1 gene and the ß-globin gene using fluorescein-labelled oligonucleotide primers. The amplicons were fractionated by capillary electrophoresis.

More specifically, a PCR reaction mixture containing PCR buffer, $MgCl_2$, dNTPs, $(NH_4)_2SO_4$, specific sense primer, specific antisense primer and heatstable polymerase was prepared. To this PCR reaction mixture was added the sample DNA and the resulting mixture was subjected to PCR carried out in the thermocycler Perkin Elmer 9700.

The PCR products were loaded onto a 2% agarose gel. After the capillary electrophoresis (Genetic-Analyzer ABI 310), the quotient of the area integral for mdr1 and the area integral for ß-globin was formed for the cancer cell fraction and the control cell fraction. There was mdr1 amplification in the cancer cell fraction if the quotient was significantly larger than that of the control.

4. Detection of ß-tubulin (mt)

Whether or not there was a point mutation at position 1092 (mutation A) or 810 (mutation B) in the ß-tubulin gene was determined by PCR-based amplification using fluorescein-labelled muation-specific oligonucleotide primers.

More specifically, a PCR reaction mixture containing PCR buffer, $MgCl_2$, dNTPs, $(NH_4)_2SO_4$, specific sense primer, specific antisense primer and heatstable polymerase was prepared. To this PCR reaction mixture was added sample cDNA and the resulting mixture was subjected to PCR carried out in the thermocycler Perkin Elmer 9700.

The following mutation A-specific primers were used:

Then, the PCR products were digested with restriction enzyme BsmI (Boehrinher Mannheim, Germany). Depending on the amount of the PCR product, a 3% MetaPhor gel or an ABI 310 Genetic analyser was used for the evaluation.

Evaluation using MetaPhor agarose gel:

Restricted PCR product and sample buffer with bromophenol blue are loaded onto a 3% MetaPhor agarose gel. The electrophoresis is carried out at a constant voltage of 130. For comparison, the uncut PCR product from an arbitrary sample is also loaded onto each gel.

Detection using an ABI 310

The detection was performed using TAMRA 500 standard kit (ABI) and in accordance the manufacture's instructions.

For mutation A, the quotient of the area integral for the mutated fragment and the area integral for the wildtype fragment was formed for both the cancer cell fraction and the control cell fraction.

There was muation A in the cancer cell fraction if the quotient was significantly larger than that of the control.

For mutation B, the quotient of the area integral for the mutated fragment and the area integral for the wildtype fragment was formed for both the cancer cell fraction and the control

cell fraction.

There was muation B in the cancer cell fraction if the quotient was significantly larger than that of the control.